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14. ABSTRACT

We have described a new form of epistasis in which direct, long range, physical interactions between genes, or gene-gene interactions mediated by specialized DNA binding proteins such as CTCF, lead to modification of phenotypic read-out. Using the associated chromatin trap (ACT) and chromosome conformation capture (3C) assays which are designed to assess physical propinquity, we investigated long range interactions of the human NF1 gene that are mediated by CTCF in normal cultured cells and in cells derived from patients with neurofibromatosis. Among the genes that were physically associated with NF1 (which is on chromosome 17) was ARF4 (ADP-ribosylation factor 4, a member of the RAS superfamily involved in membrane traffic, signal transduction and organelle integrity on chromosome 3p14.3. The relative expression of ARF4 was increased in cells and tissues from patients with neurofibromatosis compared to normal cells, suggesting that the interchromosomal interactions of NF1 regulate gene expression on chromosome 3p14.3. 4. Data obtained this year suggests that ARF4 might play a role in neurofibromatosis 1 tumorigenesis. The search for novel remote gene interactions with NF1 promises to open up totally new ranges of therapeutic targets.

15. SUBJECT TERMS
neurofibromatosis; epigenetics; epistasis; long range interactions

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Table of Contents

INTRODUCTION

One of the most remarkable aspects of neurofibromatosis 1 is the great variability in the expression of the disease, in which some affected patients may have few or mild manifestations, while others may have quite severe disease. Epistasis refers to a gene interaction in which gene A interferes with the phenotypic expression of gene B, in such a way that even if gene B is the "disease gene" (e.g., *NF1*), gene A may play an important or determining role in how the disease is manifest. We have described a new form of epistasis in which direct, long range, physical interactions between genes, or gene-gene interactions mediated by specialized DNA binding proteins such as CTCF, lead to modification of phenotypic read-out.(1)

BODY

***Task 1/2*: Characterize interactions between *NF1* and *IGF2* in normal and tumor cells.**

In our previous work, we had shown that the mouse *Nf1* gene interacted with *Igf2* (2). As we reported in the Annual Report for 2006-2007, we confirmed this association in humans, demonstrating by chromosome conformation capture (3C) and FISH that the imprinting control region between *IGF2* and *H19* on chromosome 11 interacted with *NF1* on chromosome 17. Last year, we reported that *NF1's* long range interactions were abrogated and new ones were formed in cells in which there is loss of *IGF2* imprinting.

***Task 3*: Search for new *NF1*-interacting partners**

Using the ACT assay, we began our exploration of which other genes interacted with *NF1* in both normal cell lines and in cell lines derived from patients with neurofibromatosis. Using several CTCF-binding ECR regions, we have elucidated many of these interacting genes, which are located on the multiple different chromosomes. As we reported last year, we became particularly interested in the interaction of *NF1* and *ARF4* (ADP-ribosylation factor 4, a member of the RAS superfamily involved in membrane traffic, signal transduction and organelle integrity). We confirmed the ACT data which suggested a physical interaction by directly demonstrating the interaction of one *NF1* allele with one *ARF4* allele using FISH analysis.

This year, we decided to examine the role of CTCF in NF1-related long range interactions in greater detail, since it appears that CTCF and its binding sites are absolutely crucial for the development and maintenance of inter- and intra-chromosomal interactions.

Changes in NF1 expression by changing DNA conformation at CTCF binding site ECR15

Locked Nucleic Acids (LNAs) are synthetic analogs of nucleic acids that contain a bridging methlyene carbon between the 2' and 4' positions of the ribose ring. We developed a sequence-specific anti-gene molecule, called "Zorro-LNA", which binds to both strands of DNA simultaneously, and has the potential to inhibit gene transcription. Using a Zorro-LNA targeting the ECR15 CTCF binding site of the NF1 gene (Figure 1) in the GM01859 cell line led to greatly decreased expression from both NF1 alleles (Figure 2).

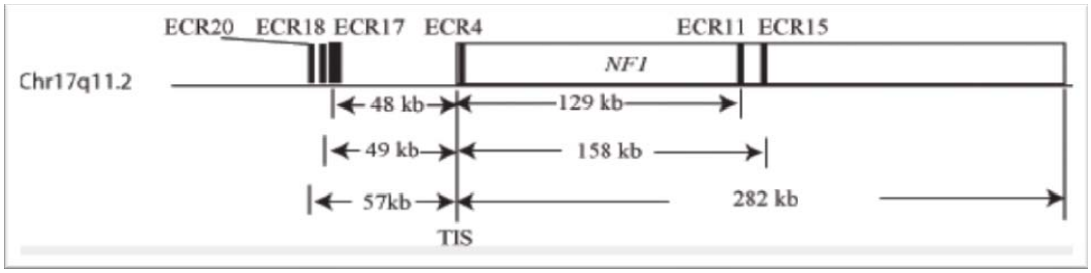

Figure 1. Schematic of *NF1* gene with various evolutionary conserved regions (ECR) noted

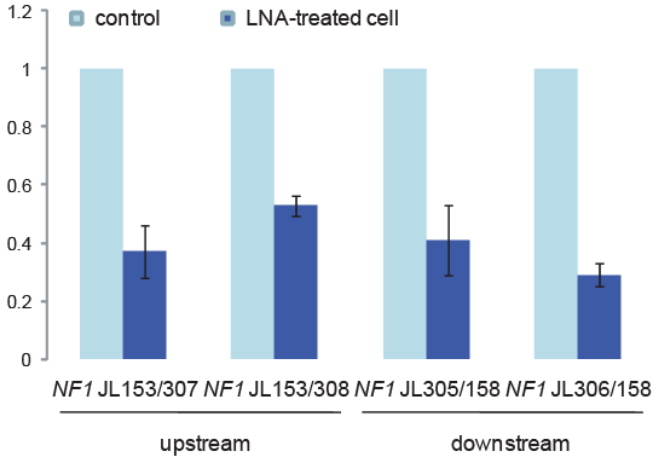

Figure 2. *NF1* gene expression in control cells and in cells treated with Zorro-LNA.

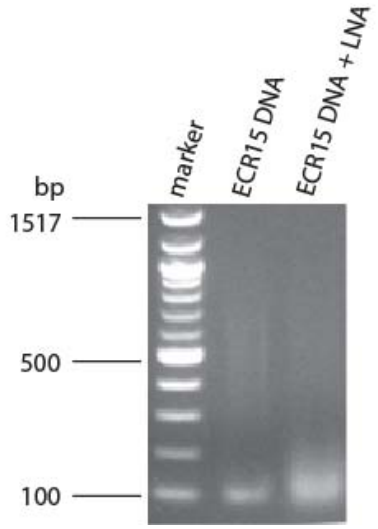

Figure 3. Conformation change in ECR15 region DNA

In vitro binding of Zorro-LNA at the ECR15 region showed a smeared band pattern in agarose gel, suggesting that there was an ECR15 region DNA conformation change after Zorro-LNA binding (Figure 3). CTCF binding was also changed in this region, as shown by EMSA (Figure 3).

Furthermore, long-range interactions between the ECR15 and the ECR4 region, which is located in promoter and exon1 region of NF1 gene, and ECR11 region, which is 30 kb upstream of ECR15 region, were changed in Zorro-LNA-treated GM01859 cells. A de novo interaction between ECR15 and ECR4 was detected in Zorro-LNA-treated GM01859 cells, while the previously seen interaction between ECR15 and ECR11 was lost in the Zorro-LNA- treated cells (Figure 4). These data show that Zorro-LNA targeting of the ECR15 CTCF binding site may alter DNA long-range interactions and result in lower gene expression of NF1.

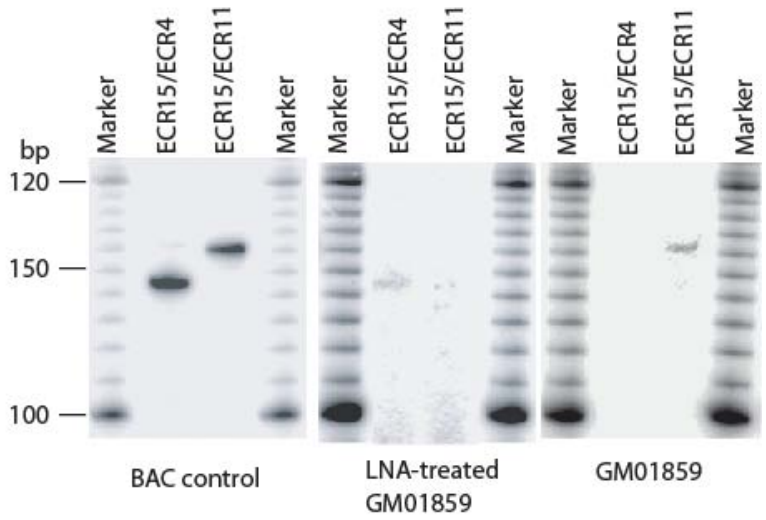

Figure 4. Chromosome conformation capture (3C) experiment demonstrating a physical interaction between regions ECR15 and ECR11 in normal cells. When the cells are exposed to the Zorro-LNA, this interaction disappears and a novel ECR15-ECR4 interaction appears.

When the Zorro-LNA targeted ECR15 region was amplified from Zorro-LNA-treated cells and cloned, sequencing revealed that the DNA sequence was unchanged in the target region (data not shown). Chromatin immunoprecipitation (ChIP) assays using CTCF and RNA Pol II antibodies showed altered protein binding of CTCF and Pol II at the ECR4 and ECR15 regions in treated cells (Figure 5), but no obvious protein binding changes in other ECR regions, such as ECR17, ECR18 and ECR20 (data not shown).

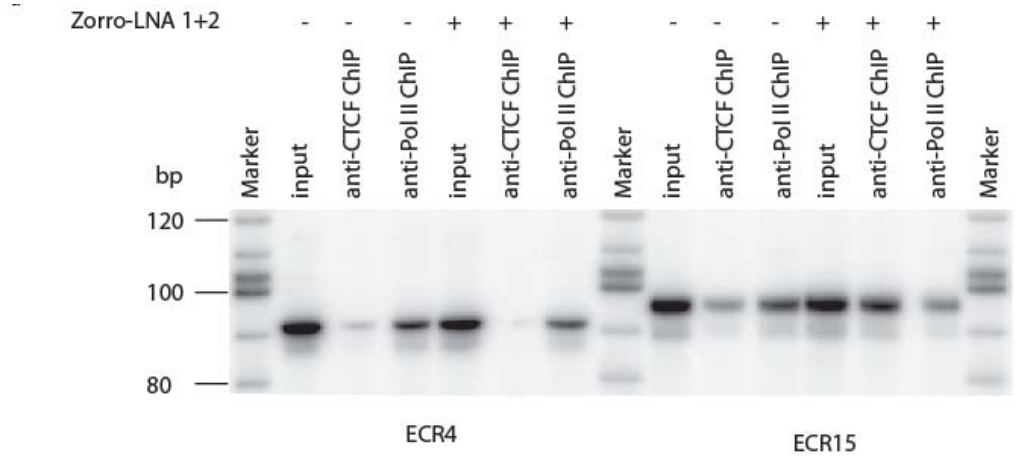

Figure 5. Altered protein-DNA interactions in Zorro-LNA treated cells

These in vivo DNA-protein interaction data show that the changes in *NF1* expression induced by Zorro-LNA are the result of changing DNA conformation and long-range DNA interactions without changes in DNA sequence. These data also show that the regulation of *NF1*expression is under the control of its nuclear architecture through long-range DNA interactions. This technique provides us a novel and efficient tool to manipulate gene expression and study gene function in disease development.

KEY RESEARCH ACCOMPLISHMENTS

- We have devised a simple method to alter long range interactions between chromatin regions.
- Changes in long range interactions are associated with changes in gene expression.
- CTCF is an important regulator of long range interactions.

REPORTABLE OUTCOMES

No new publications during this reporting period

CONCLUSIONS

1. When mutations in *NF1* occur, long range interactions may be altered, leading to changes in gene expression.
2. The relevance of these long range gene interactions in regard to the clinical manifestations of neurofibromatosis 1 needs to be investigated.
3. The search for novel remote gene interactions with *NF1* promises to open up totally new ranges of therapeutic targets.

REFERENCES

1. Ling JQ and Hoffman AR. Epigenetics of long-range chromatin interactions. Pediatr Res 61:11R-16R, 2007.
2. Ling JQ, Li T, Hu JF, Vu TH, Chen HL, Qiu XW, Cherry AM and Hoffman AR. CTCF mediates interchromosomal colocalization between Igf2/H19 and Wsb1/Nf1. Science 312:269-272, 2006.

APPENDICES: none